FORM PTO-1390 (REV 10-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

ATTORNEY'S DOCKET NUMBER: 7024491PUR92

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

U.S. APPLICATION NO. (If known, see 37 CFR 1.5): 09/700869

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US99/10821	May 18, 1999	May 18, 1998

TITLE OF INVENTION
CALCIUM CHANNEL REGULATORS

APPLICANT(S) FOR DO/EO/US
William L. PAK; Chenjian LI; Chaoxian GENG

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to promptly begin national examination procedures (35 U.S.C. 371(f)).
4. [X] The US has been elected by the expiration of 19 months from the priority date (PCT Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not communicated by the International Bureau).
   b. [ ] has been communicated by the International Bureau.
   c. [X] is not required, as the application was filed in the United States Receiving Ofice (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19(35 U.S.C. 371(c)(3))
   a. [ ] are attached hereto (required only if not communicated by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C.371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)). unsigned
10. [ ] An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 16 below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:
    A. PCT Request
    B. Resp. to International Search Rpt
    C. International Publication
    D. PCT/IB/304, 308, 332
    E. Notif. of Receipt of Demand
    F. IPER Rpt
    G. Corrected type in Abstract as suggested by IPEA

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Date of Deposit: 20 NOVEMBER 2000
I hereby certify that this correspondence is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR §1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231.
Brenda S. N. Comas
Signature of person mailing paper or fee

526 Rec'd PCT/PTO 20 NOV 2000

U.S. APPLICATION NO. (if known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/700869	PCT/US99/10821	7024491PUR92

17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)):

Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**

International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**

CALCULATIONS PTO USE ONLY	
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 100.00
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	43 - 20 =	23	X **$18.00**	$ 414.00
Independent claims	14 - 3 =	11	X **$80.00**	$ 880.00
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$270.00**	$
TOTAL OF ABOVE CALCULATIONS =				$ 1394.00
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2.				$
SUBTOTAL =				$ 1394.00
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +				$
TOTAL NATIONAL FEE =				$ 1394.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$
TOTAL FEES ENCLOSED =				$ 1394.00
			Amount to be refunded:	$
			charged:	$

a. ☒ A check in the amount of $ 1394.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 23-3030. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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SIGNATURE:

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NAME

#43,910
REGISTRATION NUMBER

09/700869
525 Rec'd PCT/PTO 20 NOV 2000

IN THE INTERNATIONAL BUREAU OF WIPO

In re PCT application of PURDUE RESEARCH FOUNDATION, etal	Authorized Officer: Gabriele Elisabeth Bugaisky
International Application Number PCT/US99/10821	Mailing Date 02 November 1999
International Filing Date 18 May 1999	Agent's File Reference:
Title of Invention CALCIUM CHANNEL REGULATORS	7024381Pur92

RESPONSE TO THE INTERNATIONAL SEARCH REPORT

The International Bureau
WIPO
34, chemin des Colombettes
1211 Geneva 20
Switzerland

Dear Sir/Madam:

In response to the International Search Report mailed 09 September 1999, regarding the above-referenced PCT Patent Application, Applicant does not wish to make any amendments at this time.

Respectfully submitted,

By _____
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PATENT & TRADEMARK OFFICE

JC09 Rec'd PCT/PTO 20 FEB 2001

PCT

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Docket No.:7024-491:117787

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	)	Before the Examiner
William L. Pak et al.	)	(Unassigned)
	)	
Serial No. 09/700,869	)	
	)	
Filing Date: November 20, 2000	)	February 14, 2001
	)	
CALCIUM CHANNEL REGULATORS	)	

SUBMITTAL OF DECLARATION / POWER OF ATTORNEY

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

Although a Notice of Missing Parts has not yet been received by Applicant, pursuant to 35 U.S.C. § 371(c)(4), Applicant files herewith their Declaration/Power of Attorney which was previously filed unsigned. A check in the amount of $130 is enclosed in accordance with 37 C.F.R. § 1.492(e). Should any other fee be required, please charge such fee to Deposit Account No. 23-3030, but not to include any payment of issue fees.

SCANNED, #4

I hereby certify that this correspondence is being deposited with the United States Postal Service as first class mail in an envelope addressed to the Assistant Commissioner for Patents, Washington, D.C. 20231 on:

February 14, 2001
(Date of Deposit)

Jason J. Schwartz
Name of Registered Representative

Signature

February 14, 2001
Date of Signature

Respectfully Submitted,

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CALCIUM CHANNEL REGULATORS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims the benefit of U.S. Provisional Patent Application Serial Number 60/087,368, filed on May 18, 1998, and U.S. Provisional Patent Application Serial Number 60/098,072, filed on August 27, 1998, both of which are hereby incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

In many types of excitable and nonexcitable cells, Ca^{2+} is both a critical molecule for homeostasis and an intracellular signaling molecule in many physiological processes such as muscle contraction, glandular secretion, transcriptional activation, and neurotransmitter release [Berridge, M.J. (1993) *Nature* 361: 315-325; Berridge, M.J. (1995) *Biochem. J.*, 312:1-11; Clapham, D.E. (1995) *Cell* 80:259-268; Clapham, D.E. (1996) *Neuron* 16:1069-1072]. Mobilization of Ca^{2+} is also involved in the immune response, such as autoimmune diseases and generation of an immune response after organ transplantation. Furthermore, a growing body of evidence suggests that neuronal degeneration diseases such as Alzheimer's is caused by excessive Ca^{2+} mobilization. These physiological processes are controlled by regulation of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). In resting cells, the cytosolic $[Ca^{2+}]_i$ is maintained at about 10-100 nM,

but during stimulation the cytosolic $[Ca^{2+}]_i$ can rise rapidly to micromolar ranges.

The widely used signal transduction pathway, the receptor-based, G protein-coupled, PLC-IP_3 cascade, also uses Ca^{2+} as a key signaling molecule. In excitable cells such as muscle cells, Purkinje neurons and *Drosophila* photoreceptor cells, as well as in nonexcitable cells such as mast cells and lymphocytes, extracellular stimuli activate receptors on the cell membrane, which in turn activate receptor-coupled G proteins. The activated G protein then activates phospholipase C to hydrolyze PIP_2 to IP_3 and DAG. While DAG activates phosphokinases, IP_3 binds to IP_3 receptors, which are ligand-gated Ca^{2+} channels on the surface of intracellular Ca^{2+} stores, and induces Ca^{2+} release from these stores. The Ca^{2+} release from intracellular stores triggers, through unknown molecules and mechanisms, Ca^{2+} influx from the extracellular space into the cell via Ca^{2+} selective channels on the plasma membrane (reviewed by Berridge, 1995; Clapham, 1996, both cited above).

Putney, in *Cell Calcium* 11:611-624 (1990), proposed that activation of the Ca^{2+} channel on the plasma membrane is dependent on Ca^{2+} release from the intracellular stores, and named these specific types of Ca^{2+} channels on the plasma membrane "capacitative Ca^{2+} channels". In recent years, "capacitative Ca^{2+}

channels" has been renamed "store-operated Ca^{2+} channels (SOC)" because, unlike the capacitors in electronic circuitry, the Ca^{2+} channels on the cell membranes actually pass ions through them. Cells throughout the animal kingdom, as well as some bacterial, fungal and plant cells, have one or more types of calcium channels.

Although physiological and pharmacological studies identified the SOCs as a unique and important class of Ca^{2+} channels, no actual genes or proteins had been identified until the *Drosophila trp* gene was cloned and subsequently studied [Montell, C. and Rubin, G.M. (1989) *Neuron* 2:1313-1323; Wong, F.E.L. et al. (1989) *Neuron* 3:81-94; Hardie, R.C. and Minke, B. (1992) *Neuron* 8:643-651; Vaca, L. et al. (1994) *Am.J. Physiol.* 267:C1510-C1505]. Several lines of research have subsequently confirmed that the Drosophila Trp protein is a member of the SOCs. Since identification of the trp gene in Drosophila, several human and mouse homologs have been cloned [Wes, P.D. et al. (1995) *Proc. Natl. Acad. Sci. USA* 92:9652-9656; Zhu, X. et al. (1995) *FEBS Letter* 373:193-198; Zitt, C. et al.(1996) *Neuron* 16:1189-1196]. Expression of the human Trp in COS cells increases store-operated calcium entry (SOCE), and expression of portions of mouse *trp* homologs in antisense orientation in murine L cells suppressed SOCE [Zhu, X. et al. (1995) above].

Further studies have determined that another protein, InaD, binds to the *Drosophila* Trp protein. InaD is a soluble protein with PDZ domains which are

known to be important for protein/protein interaction and anchoring ion channels [Kim, E. et al. (1995) *Nature* 378:85-88; Kim, E. et al. (1996) *Neuron* 17:103-113; Kormau, H.C. et al. (1995) *Science* 269:1737-1740]. InaD has been shown by co-immunoprecipitation and gel-overlay assays to bind physically to the trp protein (Shieh, B. and Zhu, M. (1996), *Neuron* 16:991-998; Huber, A. et al. (1996) *EMBO* 15(24);7036-7045]. It now appears that InaD forms the scaffold for a multimolecular signaling complex that includes the TRP protein. [Chevesich, J. et al. (1997) *Neuron* 18:95-105; Tsunoda, S. et al. (1997) *Nature* 388:243-249].

InaC has been identified as an eye-specific protein kinase C (Smith, D.P. et al. (1991) *Science* 254:1478-1484). InaC binds to InaD, suggesting that InaD could be one of the substrates of InaC-mediated phophorylation (Huber et al. (1996) above).

Although some information regarding regulation of calcium ion influx into a cell is known in Drosophila and higher eukaryotes, such as mice and humans, identification of other proteins involved in Ca^{2+} mobilization would increase the understanding of how calcium channels are regulated. Identification of proteins involved in calcium channel regulation in lower eukaryotes can lead to identification of similar proteins in higher eukaryotes, such as humans as discussed above for the trp protein. Moreover, identification of such proteins can lead to the identification of substances that modulate the activity of calcium channels, thus making it possible to treat diseases that are thought to involve calcium ion

mobilization, including Alzheimer's disease and autoimmune diseases. There is therefore a need for proteins and nucleic acid sequences involved in Ca^{2+} mobilization. The present invention addresses this need.

SUMMARY OF THE INVENTION

A novel protein, InaF, that functions in regulation of calcium ion entry into a cell, has been discovered. Accordingly, in one aspect of the invention, purified InaF proteins are provided.

In yet another aspect of the invention, isolated nucleic acid molecules that encode InaF proteins are provided. The nucleic acid molecules may be incorporated into a vector to form a recombinant nucleic acid molecule. Moreover, such recombinant nucleic acid molecules may be introduced into a host cell.

In other aspects of the invention, methods of expressing InaF proteins are provided. The methods include transforming a host cell with a nucleotide sequence encoding a protein that functions in regulating calcium ion entry into a cell as provided herein, and culturing the transformed host cells under conditions effective in achieving expression of InaF proteins. The proteins may then be purified by conventional techniques.

It is an object of the invention to provide purified functional InaF proteins.

It is a further object of the invention to provide nucleotide sequences encoding functional InaF proteins.

It is a further object of the invention to provide recombinant vectors that include nucleotide sequences encoding functional InaF proteins.

It is yet another object of the invention to provide host cells containing introduced nucleotide sequences encoding functional InaF proteins.

It is a further object of the invention to provide nucleotide sequences encoding functional InaF proteins and purified functional InaF proteins that may be modified to control calcium ion entry into cells.

These and other objects and advantages of the present invention will be apparent from the descriptions herein.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 depicts the cross scheme of single P local hopping mutagenesis for P69 and trol. Asterisks indicate chromosomes into which the P element could have transposed.

FIG. 2 depicts electroretinogram (ERG) recordings from inaF mutants as discussed in Example 2. The top trace is an ERG of strong allele, *inaF*^{P111x}, and the bottom trace is an ERG of weak allele, *inaF*^{P112x}. Stimulus duration was 4 seconds.

FIG. 3 depicts intracellularly recorded photoreceptor potentials as discussed in Example 2. The voltage responses to 8 second light stimuli were measured in wild-type flies, trp^{P301}, and inaF^{P111x}.

FIG. 4 depicts intracellularly recorded receptor potentials showing photoreceptor response latency as discussed in Example 2. Flies were dark-adapted for 2 minutes.

FIGS. 5A-B are views of photoreceptors obtained by transmission electron microscopy as discussed in Example 3. FIG. 5A, left panel, depicts photoreceptors of wild-type flies; FIG. 5A, right panel, depicts photoreceptors of 19 day old inaF;bw;st reared light/dark; FIG. 5B depicts an enlarged view of the region indicated by the arrow in FIG. 5A.

FIG. 6 depicts the cross-scheme for remobilization of the P insertion in $inaF^{P105P}$, as discussed in Example 4.

FIG. 7 depicts a cytogenetic map of the inaF mutation as discussed in Example 5. Df(1)HA85($inaF^{-}$), Df(1)m259-4($inaF^{-}$) and Df(1)($inaF^{-}$) are deficiency stocks as discussed in Example 5.

FIG. 8 depicts a genomic Southern analysis as discussed in Example 6. Genomic DNA was purified and digested by EcoRI (lanes 1-4), BamHI (lanes 5-8), and HindIII (lanes 9-12), and loaded on a 0.7% agarose gel in the following order: wild-type (lanes 1, 5 and 9); mutator 3B (lanes 2, 6 and 10); mutator 3B1-2 (jumpstarter)(lanes 3, 7 and 11); and $inaF^{P105P}$ (lanes 4, 8 and 12). The gel blot was probed with ^{32}P-dCTP labeled pCaSpeR3.

FIG. 9 depicts a genomic Southern analysis as discussed in Example 6. Genomic DNA was purified and digested by EcoRI (lanes 1 and 2), BamHI (lanes 3 and 4), and HindII (lanes 5 and 6), and loaded on a 0.7% agarose gel in the following order: mutator 3B (lanes 1, 3 and 5), and $inaF^{P105P}$ (lanes 2, 4 and 6).

FIG. 10 depicts a polytene chromosome after an in situ hybridization procedure performed as described in Example 7. The signal (arrowhead) detected on the polytene chromosome was localized in the 10 C2-E3 region of the X chromosome, which was consistent with

the results obtained by using pCaSpeR3 and fragment 4 of A23 as probes.

FIG. 11 depicts a Northern blot probed with cDNA #1 insert as discussed in Example 7. The lanes were loaded, from left to right, with polyA+ RNA from wild-type head, wild-type body, *inaF* (*inaF*^{P105P}) head and *eya* head. RP49, a ribosomal protein universally expressed in all tissues.

FIG. 12 depicts restriction maps of *inaF* cDNA and of the corresonding genomic region in the A23 clone and three inaF mutants. The unfilled inverted triangle in the *inaF*^{P105P} map identifies the P element insertion. The empty spaces to the right and left of the P insertion site in the *inaF*^{P106x} and *inaF*^{P111x} maps, respectively, represent the deletions caused by imprecise excision of the P element. In the cDNA map, the broken dotted line indicates the extent of the intron, and the open rectangle identifies the open reading frame. A composite genomic map at the top shows *Eco*RI sites (R) and the sizes of *Eco*RI fragments.

FIG. 13 depicts a Western blot analysis of null (*inaF*^{P106x}, *trp*^{P343}) and near-null (*inaF*^{P105P}, *trp*^{P301}) *inaF* and *trp* mutants, and wild-type and revertant controls. The seven lanes were loaded with total protein prepared from (lanes 1-7): wild-type heads, wild-type bodies, revertant heads, *trp*^{P301} heads, *trp*^{P343} heads, *inaF*^{P105P} heads, and *inaF*^{P106X} heads.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to preferred embodiments and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations and further modifications of the invention, and such further applications of the principles of the invention as illustrated herein, being contemplated as would normally occur to one skilled in the art to which the invention relates.

A novel calcium channel regulator protein, InaF, has been identified in the fruit fly, *Drosophila melanogaster*. Accordingly, the present invention provides purified InaF protein. The invention further provides isolated nucleic acid molecules that include nucleotide sequences encoding functional InaF proteins. Recombinant nucleic acid molecules are also provided that include the novel inaF nucleotide sequence. The nucleic acid molecules may be incorporated in a host cell. In another aspect of the invention, methods of expressing functional InaF protein are also provided.

In a first aspect of the invention, novel, purified InaF proteins are provided that function in regulating cellular influx of calcium ions. The InaF polypeptides are substantially pure (i.e., InaF proteins are essentially free, e.g., at least about 95% free, from other proteins with which they naturally occur). In one preferred embodiment, the amino acid

sequence of an InaF protein, originally found in *Drosophila melanogaster*, is set forth in SEQ ID:1.

Although the invention is described with reference to *Drosophila melanogaster* amino acid sequences, it is understood that the invention is not limited to the specific amino acid sequences set forth in SEQ ID:1. Skilled artisans will recognize that, through the process of mutation and/or evolution, polypeptides of different lengths and having differing constituents, e.g., with amino acid insertions, substitutions, deletions, and the like, may arise that are related to, or sufficiently similar to, a sequence set forth herein by virtue of amino acid sequence homology and advantageous functionality as described herein. The term "InaF protein" is used to refer generally to a protein having the features described herein and a preferred example includes a polypeptide having the amino acid sequence of SEQ ID NO:1. Also included within this definition, and in the scope of the invention, are variants of the polypeptide which function in regulating calcium ion movement into a cell, as described herein.

It is well known that animals of a wide variety of species commonly express and utilize homologous proteins, which include the insertions, substitutions and/or deletions discussed above, and yet which effectively provide similar function. For example, an amino acid sequence isolated from another species may differ to a certain degree from the sequences set forth in SEQ ID NOS:1 and 2, and yet have similar functionality with respect to catalytic and regulatory

function. Amino acid sequences comprising such variations are included within the scope of the present invention and are considered substantially or sufficiently similar to a reference amino acid sequence. Although not being limited by theory, it is believed that the identity between amino acid sequences that is necessary to maintain proper functionality is related to maintenance of the tertiary structure of the polypeptide such that specific interactive sequences will be properly located and will have the desired activity. Although it is not intended that the present invention be limited by any theory by which it achieves its advantageous result, it is contemplated that a polypeptide including these interactive sequences in proper spatial context will have good activity, even where alterations exist in other portions thereof.

In this regard, an InaF protein variant is expected to be functionally similar to that set forth in SEQ ID NO:1, for example, if it includes amino acids which are conserved among a variety of species or if it includes non-conserved amino acids which exist at a given location in another species that expresses a functional InaF protein.

Another manner in which similarity may exist between two amino acid sequences is where a given amino acid of one group (such as a non-polar amino acid, an uncharged polar amino acid, a charged polar acidic amino acid or a charged polar basic amino acid) is substituted with another amino acid from the same amino acid group. For example, it is known that the uncharged polar amino acid serine may commonly be

substituted with the uncharged polar amino acid threonine in a polypeptide without substantially altering the functionality of the polypeptide. If one is unsure whether a given substitution will affect the functionality of the enzyme, then this may be determined without undue experimentation using synthetic techniques and screening assays known in the art.

The invention therefore also encompasses amino acid sequences similar to the amino acid sequences set forth herein that have at least about 30% identity thereto and function in regulating cellular influx of calcium ions. Preferably, inventive amino acid sequences have at least about 50% identity to these sequences, further preferably at least about 70% identity, more preferably at least about 80% identity and most preferably at least about 90% identity.

Percent identity may be determined, for example, by comparing sequence information using the advanced BLAST computer program, version 2.0.8, available from the National Institutes of Health. The BLAST program is based on the alignment method of Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 87:2264-68 (1990) and as discussed in Altschul, et al., *J. Mol. Biol.* 215:403-10 (1990); Karlin and Altschul, *Proc. Natl. Acad. Sci. USA* 90:5873-7 (1993); and Altschul et al. (1997) *Nucleic Acids Res.* 25:3389-3402. Briefly, the BLAST program defines identity as the number of identical aligned symbols (i.e., nucleotides or amino acids), divided by the total number of symbols in the shorter of the two sequences. The program may be used

to determine percent identity over the entire length of the proteins being compared. Preferred default parameters for the BLAST program, blastp, include: (1) description of 500; (2) Expect value of 10; (3) Karlin-Altschul parameter λ = 0.270; (4) Karlin-Altschul parameter K = 0.0470; (5) gap penalties: Existence 11, Extension 1; (6) H value = $4.94e^{-324}$; (6) scores for matched and mismatched amino acids found in the BLOSUM62 matrix as described in Henikoff, S. and Henikoff, J.G. (1992) *Proc. Natl. Acad. Sci. USA* 89:10915-10919;Pearson, W.R. (1995) *Prot. Sci.* 4:1145-1160; and Henikoff, S. and Henikoff, J.G. (1993) *Proteins* 17:49-61. The program also uses an SEG filter to mask-off segments of the query sequence as determined by the SEG program of Wootton and Federhen (1993) *Computers and Chemistry* 17:149-163.

In another aspect of the invention, isolated nucleic acid molecules, originally isolated from *Drosophila melanogaster*, are provided that encode a functional InaF protein that functions in regulating calcium ion entry into cells. The nucleotide sequences are set forth in SEQ ID NOS:1 and 2. It is preferred that the nucleotide sequence includes nucleotides spanning nucleotides 301 to 1036 in SEQ ID NO:1 and nucleotides spanning nucleotides 528 to 1250 in SEQ ID NO:2. It is not intended that the present invention be limited to these exemplary nucleotide sequences, but include sequences having substantial similarity thereto and sequences which encode variant forms of functional InaF protein as discussed above and as further discussed below.

The term "isolated nucleic acid," as used herein, is intended to refer to nucleic acid which is not in its native environment. For example, the nucleic acid is separated from other contaminants that naturally accompany it, such as proteins, lipids and other nucleic acid sequences. The term includes nucleic acid which has been removed or purified from its naturally-occurring environment or clone library, and further includes recombinant or cloned nucleic acid isolates and chemically synthesized nucleic acid.

The term "nucleotide sequence," as used herein, is intended to refer to a natural or synthetic linear and sequential array of nucleotides and/or nucleosides, including deoxyribonucleic acid and ribonucleic acid, and derivatives thereof. The terms "encoding" and "coding" refer to the process by which a nucleotide sequence, through the mechanisms of transcription and translation, provides the information to a cell from which a series of amino acids can be assembled into a specific amino acid sequence to produce a functional polypeptide, such as, for example, an active enzyme or other protein that has a specific function. The process of encoding a specific amino acid sequence may involve DNA sequences having one or more base changes (i.e., insertions, deletions, substitutions) that do not cause a change in the encoded amino acid, or which involve base changes which may alter one or more amino acids, but do not eliminate the functional properties of the polypeptide encoded by the DNA sequence.

It is therefore understood that the invention encompasses more than the specific exemplary nucleotide

sequence of *inaF*. For example, nucleic acid sequences encoding variant amino acid sequences, as discussed above, are within the scope of the invention. Modifications to a sequence, such as deletions, insertions, or substitutions in the sequence, which produce "silent" changes that do not substantially affect the functional properties of the resulting polypeptide molecule are expressly contemplated by the present invention. For example, it is understood that alterations in a nucleotide sequence which reflect the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given site, are contemplated. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine, or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product.

Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the encoded polypeptide molecule would also not generally be expected to alter the activity of the polypeptide. In some cases, it may in fact be desirable to make mutations in the sequence in order to study the effect of alteration on the biological activity of the

polypeptide. Each of the proposed modifications is well within the routine skill in the art.

In one preferred embodiment, the nucleotide sequence has substantial similarity to the sequence set forth in SEQ ID:1 or SEQ ID:2, preferably the sequence spanning nucleotides 314 to 1036 in SEQ ID:1 and preferably the sequence spanning nucleotides 528 to 1250 in SEQ ID:2, and variants described herein. The term "substantial similarity" is used herein with respect to a nucleotide sequence to designate that the nucleotide sequence has a sequence sufficiently similar to a reference nucleotide sequence that it will hybridize therewith under moderately stringent conditions. This method of determining similarity is well known in the art to which the invention pertains. Briefly, moderately stringent conditions are defined in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd ed. Vol. 1, pp. 101-104, Cold Spring Harbor Laboratory Press (1989) as including the use of a prewashing solution of 5X SSC (a sodium chloride/sodium citrate solution), 0.5% sodium dodecyl sulfate (SDS), 1.0 mM ethylene diaminetetraacetic acid (EDTA) (pH 8.0) and hybridization and washing conditions of 55°C, 5x SSC. A further requirement of the inventive polynucleotide is that it must encode a polypeptide having similar functionality to the InaF protein described herein, i.e., functioning to regulate influx of calcium ions into cells.

In yet another embodiment, nucleotide sequences having selected percent identities to specified regions of the nucleotide sequence set forth in SEQ ID:1 are

provided. In one preferred form, nucleotide sequences are provided that have at least about 60% identity, more preferably at least about 80% identity, and most preferably at least about 90% identity, to a nucleotide sequence of substantial length within the nucleotide sequence from nucleotides 314 to 1036 set forth in SEQ ID:1. For example, such length may be 100, 200 or 400 nucleotides, or may be the entire sequence from nucleotides 314 to 1036 of SEQ ID:1. A further requirement is that the nucleotide sequence from nucleotide 314 to 1036 set forth in SEQ ID:1 encodes a protein that functions in regulating calcium entry into cells. The percent identity may be determined, for example, by comparing sequence information using the advanced BLAST computer program, version 2.0.8., as described above with reference to amino acid identity. Preferred default parameters for blastn include: (1) Karlin-Altschul parameter λ = 1.37 (gapped and ungapped); (2) Karlin-Altschul parameter K = 0.711 (gapped and ungapped); (3)H = $4.94e^{-324}$ (gapped and zero for ungapped); (4) gap penalties: Existence 5, Extension 2; and (5) scores for matched and mismatched nucleotides found in the blastn matrix as described in Altschul, S.F. et al. (1997) *Nucleic Acids Res.* 25:3389-3402 and Zhang, J. (1997) *Genome Res.* 7:649-656.

A suitable DNA sequence may be obtained by cloning techniques using cDNA libraries. For example, *Drosophila melanogaster* head cDNA libraries are available commercially or may be constructed using standard methods known in the art. Suitable nucleotide

sequences may be isolated from DNA libraries obtained from a wide variety of species by means of nucleic acid hybridization or polymerase chain reaction (PCR) procedures, using as probes or primers nucleotide sequences selected in accordance with the invention, such as those set forth in SEQ ID:1, nucleotide sequences having substantial similarity thereto, or portions thereof.

Alternately, a suitable sequence may be made by techniques which are well known in the art. For example, nucleic acid sequences encoding a functional InaF protein may be constructed by recombinant DNA technology, for example, by cutting or splicing nucleic acids using restriction enzymes and DNA ligase. Furthermore, nucleic acid sequences may be constructed using chemical synthesis, such as solid-phase phosphoramidate technology. PCR may be used to increase the quantity of nucleic acid produced. Moreover, if the particular nucleic acid sequence is of a length which makes chemical synthesis of the entire length impractical, the sequence may be broken up into smaller segments which may be synthesized and ligated together to form the entire desired sequence by methods known in the art.

In another aspect of the invention, InaF polypeptides functioning in regulating calcium ion entry into a cell and having the amino acid sequences encoded by nucleotide sequences having substantial similarity to the nucleotide sequences described above are also provided.

In a further aspect of the invention, recombinant nucleic acid molecules, or recombinant vectors, are provided. In one embodiment, the nucleic acid molecules include a nucleotide sequence encoding a functional InaF protein. The nucleotide sequence has substantial similarity, as defined above, to the nucleotide sequence set forth in SEQ ID:1 or SEQ ID:2, preferably the sequence spanning nucleotides 314 to 1036 in SEQ ID:1 or the identical sequence in SEQ ID:2 spanning nucleotides 528 to 1250. The protein produced has the amino acid sequence set forth in SEQ ID:1, or variants thereof as described above.

Recombinant vectors may be constructed by incorporating the desired nucleotide sequence within a vector according to methods well known to the skilled artisan and as described for example, in Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Laboratory, Cold Springs Harbor, New York (1982). A wide variety of vectors are known that have use in the invention. For example, various plasmid and phage vectors are known that are ideally suited for use in the invention. For example, pGEM, pBluesript, EMBL and λGt11 may be used in the invention. In one embodiment, the desired recombinant vector may be constructed by ligating DNA linker sequences to the 5' and 3' ends of the desired nucleotide insert, cleaving the insert with a restriction enzyme that specifically recognizes sequences present in the linker sequences and the desired vector, cleaving the vector with the same restriction enzyme, mixing the cleaved vector with

the cleaved insert and using DNA ligase to incorporate the insert into the vector as known in the art.

The vectors may include other nucleotide sequences, such as those encoding selectable markers, including those for antibiotic resistance or color selection. The vectors also preferably include a promoter nucleotide sequence. The desired nucleic acid insert is preferably operably linked to the promoter. A nucleic acid is "operably linked" to a another nucleic acid sequence, such as a promoter sequence, when it is placed in a specific functional relationship with the other nucleic acid sequence. The functional relationship between a promoter and a desired nucleic acid insert typically involves the nucleic acid and the promoter sequences being contiguous such that transcription of the nucleic acid sequence will be facilitated. Two nucleic acid sequences are further said to be operably linked if the nature of the linkage between the two sequences does not (1) result in the introduction of a frame-shift-mutation; (2) interfere with the ability of the promoter region sequence to direct the transcription of the desired nucleotide sequence, or (3) interfere with the ability of the desired nucleotide sequence to be transcribed by the promoter sequence region. Typically, the promoter element is generally upstream (i.e., at the 5' end) of the nucleic acid insert coding sequence.

A wide variety of promoters are known in the art, including cell-specific promoters, inducible promoters, and constitutive promoters. The promoters may further be selected such that they require activation by

activating elements known in the art, so that production of the protein encoded by the nucleic acid sequence insert may be regulated as desired.

The vectors may further include other regulatory elements, such as enhancer sequences, which cooperate with the promoter to achieve transcription of the nucleic acid insert coding sequence. By "enhancer" is meant nucleotide sequence elements which can stimulate promoter activity in a cell, such as a bacterial or eukaryotic host cell.

Moreover, the vectors may include another nucleotide sequence insert that encodes a protein that may aid in purification of the desired protein encoded by the desired nucleotide sequence. The additional nucleotide sequence is positioned in the vector such that a fusion, or chimeric, protein is obtained. For example, an InaF protein may be produced having at its C-terminal end linker amino acids, as known in the art, joined to the other protein. The additional nucleotide sequence may include, for example, the nucleotide sequence encoding glutathione-S-transferase (GST). After purification procedures known to the skilled artisan, the additional amino acid sequence is cleaved with an appropriate enzyme. For example, if the additional amino acid sequence is that of GST, then thrombin is used to separate the InaF protein from GST. The InaF protein may then be isolated from the other proteins, or fragments thereof, by methods known in the art.

The inventive recombinant vectors may be used to transform a host cell. Such methods include, for

example, those described in Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Springs Laboratory, Cold Springs Harbor, New York (1982). Once the desired nucleic acid has been introduced into the host cell, the host cell may produce the inventive InaF protein, or variants thereof, as described above. Accordingly, in yet another aspect of the invention, a host cell is provided that includes the inventive recombinant vectors described above.

A wide variety of host cells may be used in the invention, including prokaryotic and eukaryotic host cells. Bacterial host cells such as *Escherichia coli*, HB 101 and XL-1 blue may be advantageously used in the present invention. Typical eukaryotic host cells include SF9, S2, NIH 3T3 and NIH 293.

In yet another aspect of the invention, methods of producing functional InaF proteins as described above are provided. In one embodiment, the method includes providing a nucleotide sequence described above, or variants thereof, that encodes a functional InaF protein that regulates calcium ion entry into cells, and introducing the nucleotide sequence into a host cell, as described above. The desired nucleotide sequence may be advantageously incorporated into a vector to form a recombinant vector. The recombinant vector may then be introduced into a host cell according to known procedures in the art. Such host cells are then cultured under conditions, well known to the skilled artisan, effective to achieve expression of the InaF polypeptide. The InaF polypeptide may then be purified using conventional techniques.

Reference will now be made to specific examples illustrating the invention described above. It is to be understood that the examples are provided to illustrate preferred embodiments and that no limitation to the scope of the invention is intended thereby.

EXAMPLE 1

Generation of inaF Mutant by P-Element Mediated Mutagenesis

This example shows the method by which *inaF* mutants were obtained through P-element mediated mutagenesis.

Drosophila Stocks

The first *inaF* mutant was generated through P-element mediated mutagenesis, as described below, on a *white*$^-$ background. The actual eye color of the mutant was light orange because the P element insertion causing the *inaF* mutation contains a *mini-white*$^+$ gene. To eliminate eye color, the original *inaF* mutant was placed in a *bw; st* background, so that *inaF; bw; st* flies would have no eye-color pigment.

In accordance with the Pak laboratory's practice of giving a P1XX number to 1st chromosome mutations and using a lower-case letter after the number to indicate the method of inducing the mutations, we designated the original *inaF* as *inaF*^{P105p}, in which the lower-case p in the superscript stands for P-insertion. In the course of a remobilization experiment to be described below, 25 new *inaF* alleles were generated due to imprecise excision. For these additional alleles we used a lower-case x in the superscript to indicate that

they were induced by imprecise excision and designated the 25 new alleles in as *inaF*^{P106x} through *inaF*^{P130x}.

The mutator, 3B, was chosen for local hopping mutagenesis, because it has an insertion in 3B1-2, which is very close to 3A3-5 where the *P69* gene is localized. This fly has a mutation in an eye-pigment gene *white*, and thus originally has a white eye color background. The actual eye color of 3B is orange, because the fly also carries a P element, pCaspeR3, which has the mini-*white*$^{+}$ gene as a marker. The shades of eye color, from dark red to light lemon, are dependent on where the insertions are. The eye color is darker when the insertion is in the vicinity of a strong enhancer, and the eye color is lighter when the insertion is close to a weaker enhancer. This location-sensitive eye color change is a very good indication of whether the P element has been mobilized to a new place.

The mutator, y w/P[lacW] was chosen for random targeting mutagenesis. This fly carries a *white*$^{-}$ mutation, and thus has a white eye color background. Its actual orange eye color is from the P[lacW], an engineered P element with the plasmid rescue feature as well as the enhancer trap capability as described in Bier et al., *Genes and Development*, 3:1273-1287 (1989).

The jumpstarter, *P3629*, carries a functional transposase gene which lacks the end inverted repeats (delta2-3). The delta2-3 is inserted on the 3rd chromosome, which also carries a visible dominant marker Sb. This visible marker is useful in indicating the presence or absence of the delta2-3.

The *trol* mutation is lethal. In the *trol* stock used in this mutagenesis, females are balanced over FM7, and males carry, by translocation, a 2D-3C segment of the X chromosome on the Y chromosome, which rescues the *trol* lethality.

In the *C(1)RM, y w/w/Y* fly stock, females have a special type of genome that contains two linked X chromosomes. These two linked chromosomes will segregate together. If a male fly is mated to *C(1)RM, y w/Y* females, all the male offspring will carry the same X chromosome as the P1 male.

Local hopping mutagenesis to target *trol/P69*

The P element mediated local hopping mutagenesis was undertaken with the aim of isolating lethal *trol* alleles or viable ERG mutants (FIG. 1). There were three generations of crosses before the mutagenesis result could be tested by ERG.

Cross I: The mutator, 3B, which carries a pCaspeR3 in *white*$^-$ background was used. Its eye-color is light orange. The jumpstarter stock carries delta2-3 on the third chromosome, which is marked with a dominant marker Sb. In each bottle, 20 mutator males and 20 jumpstarter females were combined. Parent flies in each bottle were transferred after 4-5 days to a new bottle once, and then discarded. All flies were raised at 25^{o}C.

Cross II: Among the progeny of cross I, virgin females with Sb marker were selected to mate with males

from a FM0 containing stock. The females carried both the mutator on the X chromosome, and the jumpstarter on the 3rd chromosome. In the germ line cells of these flies, the P element in the mutator could be mobilized to new chromosomal locations because of the transposase activity conferred by delta2-3. In each round of the mutagenesis, 20 bottles of cross II were set up, each containing 20 virgin females and 20 males. Parent flies were transferred once to new food after 4-5 days, and then discarded. All flies were raised at 25°C.

Cross III: From the progeny of cross II, flies were selected for remobilized pCaspeR3, by selecting for flies with changed eye color shades (presence of *white*$^{+}$). Flies were also selected against Sb-marked delta2-3, so that pCaspeR3 insertions would be stabilized.

Both male and female progeny of cross II were used:

1) Males were single-mated to *C(1)RM, y w/Y* females carrying an attached X chromosome to establish stable lines. After 7 days, male parents were scored by an electroretinogram (ERG) as described in Example 2. If the ERG showed a mutant phenotype, the line was saved for further study; and if the ERG were wild type, the line was discarded.

2) Virgin females were single-mated to *trol/W*$^{+}$*Y* males. After 7 days, parents were transferred to fresh food. The offspring of this single-female-mating have four possible genotypes, indicated as A,B,C and D in FIG. 1.

If type A flies were found, the line was discarded because the insertion obviously was not into the *trol* gene. If type A flies were not found, then the P insertion was in the *trol* gene. The D type flies were saved for further study.

Analysis

Three rounds of local hopping mutagenesis were performed. Cross II yielded about 2% of offspring that showed changes of eye color, indicating that the P element was mobilized to new chromosomal locations. Approximately $2X10^4$ F_2 flies were scored.

Virgin female F_2 flies with eye color changes were single-female-mated to *trol*/W^+Y. The offspring of this cross were scored for complementation with *trol*. Among 179 such single-female-mating lines, none was identified as a *trol* allele.

Male F_2 flies with eye color changes were single male mated to C(1) RM, y w/Y. Among the offspring of this single-male-mating, all males carried the same X chromosome as the single male parent. 1-2 males of each line were scored by ERG. In 255 such single male mating lines, one was identified as a new mutant and designated as *inaF*.

EXAMPLE 2

Electrophysiological Identification of *inaF* Phenotypes

Electroretinogram

The electroretinogram (ERG) is an extracellular measurement of the light-induced responses in the eyes. The ERGs were recorded as described in Pak, W.L. et al.

Nature 222:351-354 (1969). A xenon arc lamp (Oriel) served as the light source with an infrared filter (7CS1-75, Corning) and Wratten neutral density filters (Kodak) were used to modulate its intensity and infrared content. In most cases, flies were raised to 5 day post-eclosion for ERG recordings. In the case of the *P69;bw;st trp*^{CM} double mutant, however, 1 day old flies were used because photoreceptors in the double mutant showed massive degeneration by day 5, but no visible defects in the eye structure on day 1.

Intracellular Recording

The intracellular recording technique was performed as described in detail by Johnson, E.C. et al. (1986) *J. Gen. Physiol.* 88(5):651-673. Flies anaesthetized with CO_2 were mounted on a glass coverslip with myristic acid. A small portion (<10%) of the cornea was cut off with a vibrating razor blade. A thin layer of inert vacuum grease was applied to the cut end to prevent desiccation of the retina.

Both the reference and the recording electrodes were inserted into the eye through the cut end of the cornea. The reference electrode was a low resistance glass microelectrode filled with physiological saline and was placed into the retinal layer of the eye. The intracellular recording electrode (FHC Borosil 1.2 mm) was pulled on a vertical Narashige puller, filled with 2 M KCl, and selected for resistance ranging between 30 to 100 mega ohm. The recording electrode was inserted into the retinal layer with a Leitz micromanipulator. Penetration of a photoreceptor was done by a minute

forward movement of the electrode and a simultaneous delivery of a brief overdriving negative capacitance current to induce oscillation at the tip of the electrode. Successful penetration of a photoreceptor cell was indicated by a drop in voltage of more than 30 mV as seen on the oscilloscope and a receptor potential of more than 20 mV in response to a bright light stimulus. The preparation was dark adapted for more than 2 minutes before any further experiments.

The measured voltage was fed to a WPI preamplifier from which the signals were directed to both an oscilloscope and a digitizer (Digidata 1200, Axon Instrument). The digitized signals were filtered at 100 Hz and were recorded by Axoscope in a Pentium computer.

Analysis

In the study of *inaF*, all flies for ERG were 2-4 days posteclosion. The most obvious mutant phenotype of *inaF* revealed by ERG and intracellular recordings is that the receptor potential fails to maintain a steady-state response during light stimuli and decays rapidly toward base line (FIGS. 2 and 3).

The rate of decay was allele-dependent. Strong alleles such as $inaF^{P111x}$ caused the receptor potential to decay to base line within 4-5 seconds under bright light stimuli. Intermediate alleles such as $inaF^{P112x}$ caused slower decay, and some of them never caused complete decay to the base line even under bright light stimuli.

The rate of decay was also dependent on the light intensity (FIG. 2) and was faster under brighter light in all *inaF* alleles.

This receptor potential decay in *inaF* closely resembles the phenotype displayed by the *trp* mutant. When the strongest mutant alleles of the two genes, $inaF^{P111x}$ and trp^{P301}, were compared, $inaF^{P111x}$ caused a stronger mutant phenotype in speed and extent of receptor potential decay (FIG. 3). FIG. 3 also shows that the receptor potential of wild-type flies is maintained at a steady state.

Another mutant phenotype became evident when the latency between the light stimulus and the photoreceptor response was examined (FIG. 4). The latency is defined as the delay between the onset of the light stimulus and the beginning of photoreceptor depolarization. This delay is light intensity dependent and has been interpreted as the time required by the phototransduction pathway to proceed from photoconversion of rhodopsin to the opening of light-activated channels on the plasma membrane. In both trp^{P301} and $inaF^{P111x}$, the latency was prolonged compared to that of the wild type, and the delay was greater in $inaF^{P111x}$.

EXAMPLE 3

Effect of inaF Mutation on Retinal Degeneration

This example shows that the *inaF* photoreceptors undergo a light-dependent degeneration. Degeneration is also age-dependent and is not detectable in young (<1 week old) inaF mutants.

Transmission Electron Microscopy

The transmission electron microscopy technique was identical to the method described by Fan, S.S. and Ready, D.F. (1997) *Development* 124:1497-1507. Flies were microinjected with aldehyde fixative (2% paraformaldehyde and 1.75% glutaraldehyde in 0.1 M sodium cacodylate, pH 7.4) and dissected after 1 hour. Fixed eyes were incubated in 1% tannic acid overnight and transferred to 2% osmium tetroxide in 0.1 M sodium cacodylate for 2 hours. After washing, the eyes were incubated overnight in 2% uranyl acetate. After a serial dehydration with ethanol, eyes were mounted in Epox 812. Tissue was then sectioned using a Reichert ultramicrotome and observed using a Philips 300 electron microscope.

Analysis

Retinal degeneration was observed in *inaF* compound eyes (FIGS. 5A-B). For better control, $inaF^{P105P}$ was put in a *bw; st* background to eliminate eye color. Confocal microscopy and EM were used to examine photoreceptor structures. The photoreceptors in $inaF^{P105P}$; *bw; st* showed no detectable abnormality at 1 day posteclosion, suggesting that they had developed normally. However, flies raised in a 12 hour-light/12 hour-dark cycle to 19 days posteclosion showed retinal degeneration. Rabdomeres were absent in some ommatidia; most of the rabdomeres were much reduced in size; microvilli (membrane which contains rhodopsin) were disrupted by vacuolized structures; and the base of the microvilli was no longer smooth and regular.

To test if this retinal degeneration was light dependent, *inaF*^{P105p}; *bw; st* flies were raised in complete darkness from the embryo stage to 19 days posteclosion. EM study of these fly eyes indicated that the photoreceptor structure was largely intact (data not shown). Hence the retinal degeneration in *inaF* is light dependent.

EXAMPLE 4

Proof that the inaF Mutation is Caused by P-Element Insertion - Remobilization of the P insertion in *inaF*^{P105p}

The most direct and reliable method to demonstrate that a mutation is in fact caused by a P element insertion is to remobilize the P element. If the P element is the cause of the mutation, then one would expect two possibilities when the P insertion is removed from the genome. First, if the P element is precisely excised, then the mutated gene will be restored, and the mutant flies revert to wild type (thus they are called "revertants"). Second, if the excision is imprecise and takes away some flanking DNA with the remobilized P element, the flies will continue to show a mutant phenotype.

If, however, the mutation is actually not caused by the P insertion, but by some other defect such as a spontaneous point mutation, then the flies with the P element remobilized will always remain mutant, i.e., no wild-type revertants will be recovered.

Remobilization of the P insertion in *inaF*^{P105p}

inaF^{P105p} was used as a mutator and crossed to the jumpstarter, *P3269*. The design and protocol for this remobilization experiment are the same as those of local hopping mutagenesis described in Example 1 above, except that white-eyed flies (indicating loss of the P element which carries the *mini-white*$^{+}$ gene) were selected among the offspring of cross II (FIG. 6). These virgin females were single mated to FM0/Y males. The offspring had four possible genotypes (X- indicates an excision event):

A: X- *inaF*^{P105p} -X/FM0; +/+; +/+

B: FM0/FM0; +/+;+/+

C: FM0/Y;+/+;+/+

D: X- *inaF*^{P105p} -X/Y;+/+;+/+

Type D offspring in each single-female line were selected for ERG. If the ERG showed the *inaF* phenotype, the excision event was an imprecise one. Types A and D were saved to establish stable lines of new *inaF* alleles. If the ERG showed a wild-type response, the excision event was a precise one. Types A and D were saved to establish stable lines of these revertants. These lines were used in chromosomal *in situ* hybridization with the P element as a probe to confirm that the P insertion in *inaF*^{P105p} no longer existed.

Analysis

In cross III, 260 single-female-mating lines were set up, and their offspring were scored by ERG. We obtained 126 wild-type revertants, 61 lethal mutants, and 25 mutants showing the *ina* ERG phenotype and thus presumed to carry new mutant *inaF* alleles. Flies from these 25 lines were crossed to $inaF^{P105p}$ for complementation and confirmed to carry mutant alleles of $inaF^{P105p}$.

This result unequivocally demonstrated that the $inaF^{P105p}$ mutation is caused by a P element insertion.

EXAMPLE 5

Cytogenetic Mapping of the $inaF^{P105p}$ Mutation

To map the *inaF* mutation cytogenetically, a group of deficiency stocks were obtained from the *Drosophila* Stock Center at Indiana University and mated to the *inaF* mutant. The heterozygous F_1 flies that carried Deficiency/*inaF* chromosomes were scored by ERG.

Analysis

A group of deficiency stocks carrying deletions in the 10 C2-E3 region were used to map the $inaF^{P105p}$ mutation cytogenetically (FIG. 7). Three of them did not complement the $inaF^{P105p}$ mutation. Thus, results from cytogenetic mapping independently localized the $inaF^{P105p}$ mutation to the 10 C2-E3 region of the X chromosome, consistent with the P insertion site

identified by chromosomal *in situ* hybridization as described in Example 9 below.

EXAMPLE 6

An Eye-Specific Clone A23 Fragment Contains the inaF Gene

This example shows that, by analyzing genomic Southern and Northern blots, clone A23 was shown to contain the *inaF* structural gene.

Previous research has shown that the majority of genes important for phototransduction are expressed specifically or preferentially in the eyes. Since *inaF* is a vision defective mutant and clone A23 co-localizes with *inaF*, it is possible that clone A23 may contain the *inaF* structural gene. However, the 10 C-D-E region of the X chromosome contains about 500 kb of genomic DNA which accommodates about 50-100 genes. Therefore it is also possible that clone A23 represents an eye-specific gene in that region but is unrelated to the *inaF* gene. This question was resolved by a combination of genomic Southern and Northern analyses.

Isolation of Clone A23

Several years ago, the Pak laboratory isolated a pool of *Drosophila* eye-specific clones by subtractive hybridization. In that method, poly(A)$^+$ RNA extracted from the heads of wild type flies was reverse transcribed into cDNA and hybridized with an excess amount of poly(A)$^+$ RNA extracted from the heads of *eyes absent* (*eya*) mutant flies, all according to standard

protocols. The eye-specific, single-stranded cDNA molecules were then separated from the hybridization mixture by hydroxyapatite chromatography according to standard protocols and used to screen a genomic library to generate a pool of eye-specific clones. These clones were further confirmed by dot blots and Northern blots. The confirmed eye-specific clones were localized on the polytene chromosomes by chromosomal *in situ* hybridization. One of them, A23, was localized in the 10 D region of the X chromosome.

Genomic Southern Analysis

Genomic DNA of wild-type flies, the 3B mutators and the *inaF*^{P105P} flies was isolated by homogenizing fifteen to twenty flies and using the Puregene kit from Gentra Co. following recommended protocols. 3 µg of genomic DNA of each type was digested with restriction enzymes of choice and loaded on a 0.7% agarose gel for electrophoresis. The agarose gel was denatured in 1.5 M NaCl, 0.5 M NaOH solution for 30 minutes, neutralized in 1 M Tris-Cl, 3 M NaCl, pH 7.5 solution for 40 minutes, blotted overnight onto Hybond-N$^+$ Nylon membrane (Amersham Co.), and UV cross-linked.

1 µg of genomic or cDNA fragments was used as template for ^{32}P-dCTP labeling with random primers. The radioactively labeled probe was purified with a Sephadex G-50 column.

Prehybridization treatment was carried out in 0.5 M NaH_2PO_4, 0.7% SDS, 1% BSA, 0.01 M EDTA solution at 68^{o}C for 3-4 hours, and hybridization was carried out in the same solution at 68^{o}C for 16-20 hours. Washing was carried out in 0.04 M sodium phosphate buffer, 5%

SDS, 0.5% BSA, 0.01 M EDTA solution twice for 20 minutes and in 0.04 M sodium phosphate buffer, 1% SDS, 0.01 M EDTA solution twice for 40 minutes. Kodak X-ray film was used for autoradiography.

Northern Analysis

The poly(A)$^+$ RNA was extracted with a PolyATtract-1000 kit from Promega Co. following their recommended protocol. 3 μg of poly(A)$^+$ RNA was loaded in each lane of the agarose gel unless otherwise specified. 1 μg of genomic DNA or cDNA fragment was used as template for ^{32}P-dCTP labeling with random primers. Prehybridization, hybridization, and washing of Northern blots were carried out according to the standard protocol in Sambrook et al., *Molecular Cloning A Laboratory Manual*, 2nd ed. Vol. 1, Cold Spring Harbor Laboratory Press (1989).

Analysis

Genomic Southern analyses were used to determine whether A23 contains DNA fragments flanking the P element insertion that causes the *inaF* mutation. Since the P insertion is in *inaF*, A23 could not contain the *inaF* gene if it were far removed from the P insertion.

Genomic DNA from wild-type flies, mutator 3B, and *inaF*^{P105P} was purified and digested with multiple restriction enzymes, electrophoresed and blotted. A genomic Southern blot was probed with pCaSpeR3 (FIG. 8). Restriction fragment length polymorphism (RFLP) was observed and can be interpreted as follows: 1) The RFLP between wild type and 3B is due to an additional P element in 3B; and 2) The RFLP between 3B and

inaF^{P105p} is due to the fact that DNA fragments of different sizes flank the P element insertion sites in 3B and *inaF*, and these were detected by the ^{32}P-dCTP labeled pCaSpeR3 probe.

Other Genomic Southern blots were probed by ^{32}P-dCTP labeled A23 fragments. Among A23 fragments, fragment 4 (3.6 kb) detected RFLPs between wild-type flies, 3B, and *inaF*^{P105p} that were similar to those detected by the pCaSpeR3 probe as seen in FIG. 8, except that the EcoRI lanes showed same size signals. This could be due to the fact that the new flanking DNA sequences in *inaF*^{P105p}, though a different species, has the same size as the one flanking the P insertion in 3B. The similarity of the RFLPS between wild-type flies, 3B, and *inaF*^{P105p} indicates that fragment 4 is likely to contain DNA flanking the P insertion site in *inaF*^{P105p} (FIG. 9).

Northern blots were used to examine whether fragment 4 of A23 could detect alterations of transcripts between wild-type flies and *inaF*^{P105p}. Poly(A)$^+$ RNA was purified from wild-type fly heads, wild-type fly bodies, *eya* heads, and *inaF*^{P105p} heads. Because *inaF*^{P105p} flies undergo age-dependent retinal degeneration, and because confocal microscopy did not detect retinal degeneration in young (<5 days) *inaF*^{P105p} flies, polyA$^+$ RNA was purified from 1-3 days old *inaF*^{P105p} flies. Fragment 4 of A23 was used as a probe for Northern analysis, and detected a 3.0 kb eye-

specific transcript which was drastically reduced in *inaF*^{P105p} flies.

Results from the genomic Southern and Northern analyses jointly indicated that fragment 4 of clone A23 contained at least part of the inaF gene and possibly all of it.

EXAMPLE 7

Isolation of inaF cDNA Clones From a *Drosophila* Head cDNA Library

Screening Procedure

Fragment 4 of A23, a genomic DNA fragment, was used as a template for ^{32}P-dCTP labeling with random primers. The labeled probe was purified with a Sephadex G-50 column and was used to screen 5 X 10^5 plaque forming units (pfu) of a *Drosophila* head cDNA Library, a gift from Dr. Erich Buchner at Wurzburg University in Germany. The cDNA library screening was carried out according to a standard protocol in Sambrook et al., *Molecular Cloning A Laboratory Manual*, 2nd ed. Vol. 1, Cold Spring Harbor Laboratory Press (1989).

Analysis

10 positively hybridizing cDNA clones were obtained and purified as single plaques. Cross hybridization among these clones demonstrated that they all belong to the same class of cDNA. cDNA#1 had the biggest insert and thus was used for further experiments. The insert of cDNA#1 was labeled with

biotin-dUTP and used as a probe for chromosomal *in situ* hybridization and detected a hybridization signal in the 10 C2-E3 region as described in Example 8. The insert was also used to probe a genomic Southern blot and detected the same RFLP as those revealed by pCaSpeR3 and A23 probes. Finally, the insert was labeled with ^{32}P-dCTP and used to probe a Northern blot.

Three μg of polyA+ RNA of each sample was loaded on the gel. cDNA#1 insert was labeled with ^{32}P-dCTP. A 3.0 kb transcript was detected in the poly(A)$^+$ RNA from wild-type fly heads but not that from wild-type fly bodies and *eya* heads, indicating that the 3.0 kb transcript is eye specific (FIG. 11). The same transcript was absent from the poly(A)$^+$ RNA from inaF^{P105p}, indicating that the cDNA most likely contains the inaF gene. The same blot was boiled to eliminate the radioactive probe and used again for a control experiment in which RP49, a ribosomal protein universally expressed in all tissues, was used as a probe. These lines of evidence all suggested that cDNA#1 corresponds to the *inaF* gene.

EXAMPLE 8

Chromosomal Location of the *inaF*^{P105p} P Insertion

To localize the *inaF*^{P105p} P insertion site on polytene chromosomes, pCaSpeR3, the P element employed in the local hopping mutagenesis described in Example 1, was used as a template for synthesizing biotin-dUTP probes for chromosomal *in situ* hybridization to the

polytene chromosomes of *inaF*^{P105P}. In this case, however, a genomic DNA fragment from clone A23, discussed in Example 6, and a cDNA fragment of cDNA#1 were used as templates for the biotin-dUTP labelling. The probe hybridized to 10 C2-E3 (FIG. 10). The signal at 10 C2-E3 was due to detection of the new P insertion.

EXAMPLE 9

Sequence of *inaF* cDNA

Sequencing cDNA clone #1

cDNA clone #1 was partially digested with EcoRI and subcloned into the pBluescript-SK^{+} vector. T3 and T7 primers were used for initial sequencing reactions, and internal sequencing primers were designed and synthesized according to the sequence data obtained from each gel reading. The sequencing reactions were carried out at the DNA Sequencing Center at Iowa State University, Ames, Iowa. Both strands were sequenced, and every nucleotide has been confirmed from at least three independent reactions. The sequence of cDNA clone 1 is set forth in SEQ ID:1. A similar nucleotide sequence, differing only in certain 5' regions, and including a linker sequence at the 3' end, is set forth in SEQ ID:2.

Analysis

The cDNA has a poly(A)$^{+}$ tail immediately before the 3'-end EcoRI cloning site, and a consensus

polyadenylation signal (AATAAA) preceding the polyA$^+$. This indicates that the 3' end of the cDNA is intact.

At the 5' end, the translation start site was determined on the basis that an in-frame stop codon is present about 15 amino acids upstream to the methionine assigned as the +1 site.

The putative protein has 241 amino acids with an estimated molecular weight of 26 kd. It appears to be a soluble protein since the Kyte-Doolittle plot did not reveal any hydrophobic segments which can serve as transmembrane domains. A BLAST search of the NCBI, EMBL and SWISSPORT databases did not find significant homology with any known proteins. A MOTIF search identified two potential glycosylation sites (position 18 and 103) and a potential PKC phosphorylation site (position 144).

FIG. 12 shows a restriction map of inaF cDNA and of the corresponding genomic region in the A23 clone and three inaF mutants.

EXAMPLE 10

Immunodetection of the TRP protein

To determine if inaF mutations affect the amount of the TRP protein, Western blot analyses were performed. The blot was probed with a monoclonal anti-TRP antibody described in Pollock, *J. Neurosci.* 15:3747-3760 (1995). Results showed that the TRP protein is reduced to about 15% and 10% of the wild type level in *inaF*^{P105p} and *inaF*^{P106x}, respectively, at 1 day post-eclosion (FIG. 13). The reductions are not due to non-specific reductions of retinal proteins. Other retinal proteins examined [rhodopsin, PLCB

(NORPA), and InaD) did not show similar reductions at this age (data not shown), nor were there any signs of retinal degradation in such young flies.

EXAMPLE 11

Recombinant Expression Vectors Encoding InaF

A glutathione-S-transferase-InaF polypeptide (GST-InaF) fusion construct was made by ligating the *inaF* coding region in frame with the glutathione transferase gene in the pGEX-KG vector [(Guan and Dixon, *Anal. Biochem.*, 192:262-267 (1991)]. Following transformation of bacteria (E. coli BL-21), over expression of the fusion protein was achieved by induction with IPTG. The fusion protein was partially purified by using immobilized glutathione [Guan and Dixon (1991), cited above]. Further purification can be achieved by ion exchange chromatography. In order to obtain purified InaF protein, the fusion protein can be digested with thrombin (Sigma) and the InaF protein can be eluted from an immobilized glutathione agarose column as known in the art.

Biological Deposit Under The Budapest Treaty

A deposit of inaF cDNA, designated as inaF cDNA-1/XL-1 Blue was deposited with the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209. The deposit is Epicurian Coli XL-1 Blue (Stratagene) harboring inaF cDNA (SEQ ID:1, nucleotides 314 to 1036) from Drosophila melanogaster (Berlin) in a

pBluescript II KS (Stratagene) vector. The accession number is ATCC 207232.

While the invention has been illustrated and described in detail in the drawings and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that only the preferred embodiment has been shown and described and that all changes and modifications that come within the spirit of the invention are desired to be protected. In addition, all references cited herein are indicative of the level of skill in the art and are hereby incorporated by reference in their entirety.

CLAIMS

What is claimed is:

1. An isolated nucleic acid molecule, comprising a nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells, said nucleotide sequence having substantial similarity to the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

2. The molecule of claim 1, wherein said nucleotide sequence is comprised of the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

3. The molecule of claim 1, wherein said protein is comprised of an amino acid sequence having at least about 30% identity with the amino acid sequence set forth in SEQ ID:1.

4. The molecule of claim 1, wherein said protein is comprised of an amino acid sequence having at least about 50% identity with the amino acid sequence set forth in SEQ ID:1.

5. The molecule of claim 1, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1.

6. The molecule of claim 1, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1 or a sufficiently similar sequence thereto to exhibit the ability to regulate calcium ion entry into cells.

7. An isolated nucleic acid molecule, comprising a nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells, said nucleotide sequence having the sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

8. An isolated nucleic acid molecule, comprising a nucleotide sequence having at least about 80% identity to a 400 nucleotide long sequence within the sequence set forth in SEQ ID:1 from nucleotide 301 to nucleotide 1036, said nucleotide sequence from nucleotide 301 to nucleotide 1036 encoding a protein functioning in regulating calcium entry into cells.

9. A recombinant nucleic acid molecule, comprising a nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells, said nucleotide sequence having substantial similarity to the sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

10. The molecule of claim 9, wherein said nucleotide sequence is comprised of the sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

11. The molecule of claim 9, wherein said protein is comprised of an amino acid sequence having at least about 30% identity with the amino acid sequence set forth in SEQ ID:1.

12. The molecule of claim 9, wherein said protein is comprised of an amino acid sequence having at least about 50% identity with the amino acid sequence set forth in SEQ ID:1.

13. The molecule of claim 9, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1.

14. The molecule of claim 9, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1 or a sufficiently similar sequence thereto to exhibit the ability to regulate calcium ion entry into cells.

15. The molecule of claim 9, further comprising a promoter operably linked to a terminal 5' end of said nucleotide sequence.

16. The molecule of claim 15, wherein said promoter is selected from the group consisting of a constitutive promoter, an inducible promoter, and a cell-specific promoter.

17. A recombinant nucleic acid molecule, comprising a nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells, said nucleotide sequence having the sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

18. A host cell, comprising an introduced nucleic acid molecule having a nucleotide sequence of substantial similarity to the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036, said nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells.

19. The host cell of claim 18, wherein said nucleotide sequence is comprised of the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

20. The host cell of claim 18, wherein said protein is comprised of an amino acid sequence having at least about 30% identity with the amino acid sequence set forth in SEQ ID:1.

21. The host cell of claim 18, wherein said protein is comprised of an amino acid sequence having at least about 50% identity with the amino acid sequence set forth in SEQ ID:1.

22. The host cell of claim 18, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1.

23. The host cell of claim 18, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1 or a sufficiently similar sequence thereto to exhibit the ability to regulate calcium ion entry into cells.

24. A host cell, comprising an introduced nucleic acid molecule having a nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036, said nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells.

25. A purified InaF protein.

26. A purified protein, said protein having an amino acid sequence having at least about 30% identity to the amino acid sequence set forth in SEQ ID:1, said protein functioning in regulating calcium ion entry into cells.

27. The protein of claim 26, wherein said protein has an amino acid sequence as set forth in SEQ ID:1.

28. The protein of claim 26, wherein said protein has an amino acid sequence having at least about 50% identity to the amino acid sequence set forth in SEQ ID:1.

29. The protein of claim 26, wherein said protein has an amino acid sequence as set forth in SEQ ID:1 or a sufficiently similar amino acid sequence thereto to exhibit the ability to regulate calcium ion entry into cells.

30. A purified protein, said protein having an amino acid sequence set forth in SEQ ID:1, said protein functioning in regulating calcium ion influx into cells.

31. A purified protein, said protein having an amino acid sequence encoded by a nucleic acid molecule having a nucleotide sequence of substantial similarity to the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036, said protein functioning in regulating calcium ion entry into cells.

32. A recombinant protein, comprising: an amino acid sequence having at least about 30% identity to the amino acid sequence set forth in SEQ ID:1, said protein functioning in regulating calcium ion entry into cells.

33. The protein of claim 32, wherein said protein has an amino acid sequence having at least about 50% identity to the amino acid sequence set forth in SEQ ID:1.

34. A method of expressing an InaF protein, said method comprising:

(a) introducing into a host cell a nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells, said nucleotide sequence having substantial similarity to the sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036; and

(b) culturing under conditions to achieve expression of said protein.

35. The method of claim 34, wherein said nucleotide sequence is comprised of the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

36. The method of claim 34, wherein said protein is comprised of an amino acid sequence having at least about 30% identity with the amino acid sequence set forth in SEQ ID:1.

37. The method of claim 34, wherein said protein is comprised of an amino acid sequence having at least about 50% identity with the amino acid sequence set forth in SEQ ID:1.

38. The method of claim 34, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1.

39. The method of claim 34, wherein said protein is comprised of an amino acid sequence set forth in SEQ ID:1 or a sufficiently similar sequence thereto to exhibit the ability to regulate calcium entry into cells.

40. The method of claim 34, wherein said nucleotide sequence is inserted in a vector.

41. The method of claim 40, wherein said vector is a plasmid vector.

42. A method of expressing an InaF protein, said method comprising:

(a) introducing into a host cell a recombinant nucleic acid molecule comprising a nucleotide sequence encoding a protein functioning in regulating calcium ion entry into cells, said nucleotide sequence having substantial similarity to the sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036; and

(b) culturing under conditions to achieve expression of said protein.

43. The method of claim 42, wherein said nucleotide sequence is comprised of the nucleotide sequence set forth in SEQ ID:1 from nucleotide 314 to nucleotide 1036.

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306 cagtaactag taaccaatta ccaattacca gtaacccatc caaggagtat accccccttg      2260
308 caaacgggga agcggataaa tgtcactaga attcagcatc atcagattga atcacacaca      2320
310 atcctagtcg cctcacgcga agagaactat gtcatgatca gatatcggtg tatgcattct      2380
312 atattatgta cttcgaaata tgtaatttat taagttttcg ctatactttt cattcaaatt      2440
314 ggcaaaaacc aattcaaagg ttttcaatat tttcgaaaag cattttaggc tttctatgta      2500
316 acgtatgttt ttcaaacaaa atattagttt ttgaaacttt attatcggat aaacaaatgt      2560
318 aagccaaatt acaacgttta tgatactcca aagattcgca ctataaagtg gcctaaaaat      2620
320 agctgacgca ttagccatag gcgcttcgct tctcaagata aaacctgggc gtgctcaact      2680
322 caagaacaaa tatgtggtta tatacatata tacatatatg gggcatataa ccgatgtgtg      2740
324 acgtgacatt ggctcgttct attcacatac ttaaacacta aatgcaaacc tatcaaaaac      2800
326 caactacact aagcgaaaag cggcagagat agttaaggaa agtggtcaag agaggacgag      2860
328 agagagagag agagaaagtg aaagtgaaag ggagagatag taaaactgca tctgcatcca      2920
330 aagacacgag aattgaattc atcaataata acatacgtat aaacgatatg catacgatat      2980
332 agaattgaat ctgtaactga tgggcatata ccgcatatat atcttatata ccgcatatat      3040
334 cttatatatg tataccaaga aaaacaaagt catttggcaa taataaagca tagcaaacaa      3100
336 caataaaaaa aa                                                          3112
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VERIFICATION SUMMARY
PATENT APPLICATION: **US/09/700,869**

DATE: 08/07/2001
TIME: 17:38:40

Input Set : **A:\Pto.amc**
Output Set: **N:\CRF3\08072001\I700869.raw**

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L:11 M:270 C: Current Application Number differs, Replaced Current Application Number
L:13 M:271 C: Current Filing Date differs, Replaced Current Filing Date
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PCT09

RAW SEQUENCE LISTING
PATENT APPLICATION: US/09/700,869

DATE: 07/15/2001
TIME: 20:15:54

Input Set : A:\PUR-92SEQ.doc
Output Set: N:\CRF3\07152001\I700869.raw

W--> 1 PUR-092-PCT:46662/7024-384 v.7 delete

Does Not Comply
Corrected Diskette Needed

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 5 <110> APPLICANT: Pak, William L.
 6       Li, Chenjian
 7       Geng, Chaoxian
 9 <120> TITLE OF INVENTION: Calcium Channel Regulators
11 <130> FILE REFERENCE: 7024-384/PUR-92
C--> 13 <140> CURRENT APPLICATION NUMBER: US/09/700,869
C--> 15 <141> CURRENT FILING DATE: 2001-05-24
17 <150> PRIOR APPLICATION NUMBER: U.S. 60/087,368
19 <151> PRIOR FILING DATE: 1998-05-18
21 <150> PRIOR APPLICATION NUMBER: U.S. 60/098,072
23 <151> PRIOR FILING DATE: 1998-08-27
25 <160> NUMBER OF SEQ ID NOS: 2
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31 <211> LENGTH: 2905
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44 cacacgcatc cgattggcta ggatcggatg gctatcattt agttagccat ccatctccca      180
46 gcgacatcag tgaccggaat tggataaaat tgtgataccc gagaaaatag ccattcgcct      240
48 cctgaactca tcggaagtga ccgcggagca gttctacaag cacatcctcg agcagtaccg      300
50 catcctcagc cac atg caa cag cag cgc cag caa ctg ctg cag cgc caa cat      352
51                 Met Gln Gln Gln Arg Gln Gln Leu Leu Gln Arg Gln His
52                 1               5                  10
54 ctc caa ctg cag cag ctg gag gca aac aat cgc ttc cag gag gtc ttt        400
55 Leu Gln Leu Gln Gln Leu Glu Ala Asn Asn Arg Phe Gln Glu Val Phe
56     15                  20                  25
60 gcc acg gcc acc atc att cag gca cat ccg cat ccc cat cca cat ccc       448
61 Ala Thr Ala Thr Ile Ile Gln Ala His Pro His Pro His Pro His Pro
62 30                  35                  40                  45
64 agg gag ccg ccc aag aag ccg ctt tta gga cca tat agc ccg caa ccc       496
65 Arg Glu Pro Pro Lys Lys Pro Leu Leu Gly Pro Tyr Ser Pro Gln Pro
66                 50                  55                  60
68 ggc aac ata agt cac gct atg ggt ggt gat cag ttg gat gca gaa acg       544
69 Gly Asn Ile Ser His Ala Met Gly Gly Asp Gln Leu Asp Ala Glu Thr
70             65                  70                  75
72 gaa cag ggt cac atg cct cta atc ctg gat acc tca ccg ccg gtc gaa       592
73 Glu Gln Gly His Met Pro Leu Ile Leu Asp Thr Ser Pro Pro Val Glu
74         80                  85                  90
76 gta acc gga atg ggt cac ctg aag cgg aag aca cat cgc ggt cac tac       640
77 Val Thr Gly Met Gly His Leu Lys Arg Lys Thr His Arg Gly His Tyr
78     95                  100                 105
80 aaa cat cat aga gcc cga gcc ggt ggt caa aag aaa ctg tcc att gcc       688
81 Lys His His Arg Ala Arg Ala Gly Gly Gln Lys Lys Leu Ser Ile Ala
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RAW SEQUENCE LISTING DATE: 07/15/2001
PATENT APPLICATION: US/09/700,869 TIME: 20:15:54

Input Set : A:\PUR-92SEQ.doc
Output Set: N:\CRF3\07152001\I700869.raw

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 85 Asn Ser Met Ala Ser Ser Thr Pro Ser Thr Thr Ala Gly Gly Asp Ala
 86                 130                 135                 140
 88 tca atg gcc act gcg gcc act ttg cca cat ggt tat atg gac gct cca       784
 89 Ser Met Ala Thr Ala Ala Thr Leu Pro His Gly Tyr Met Asp Ala Pro
 90             145                 150                 155
 92 cta aat ccg gcg gca gga acc att gtc cag gca cca caa ctg cag cta       832
 93 Leu Asn Pro Ala Ala Gly Thr Ile Val Gln Ala Pro Gln Leu Gln Leu
 94         160                 165                 170
 96 tac acc tcg atg ccc att cca ctg atc ctg agt ccc agc gac gaa aag       880
 97 Tyr Thr Ser Met Pro Ile Pro Leu Ile Leu Ser Pro Ser Asp Glu Lys
 98     175                 180                 185
100 cgt cct tcg cac cac gcc cac gga cat gtc cat ggc gag agg cgg aac       928
101 Arg Pro Ser His His Ala His Gly His Val His Gly Glu Arg Arg Asn
102 190                 195                 200                 205
104 ggg gcg caa tcc ggc ggc cgg cga agg acc acg acg gca tcg gtt tct       976
105 Gly Ala Gln Ser Gly Gly Arg Arg Arg Thr Thr Thr Ala Ser Val Ser
106                 210                 215                 220
108 ggc tac gag gcg cag acc tac ctc aat ccg ttt ctc acc ggc gag ctg      1024
109 Gly Tyr Glu Ala Gln Thr Tyr Leu Asn Pro Phe Leu Thr Gly Glu Leu
110             225                 230                 235
112 atc ttc gag aag taagggactg cacccagatc aggaaacgtc gcggttcatt          1076
113 Ile Phe Glu Lys
114         240
116 gttttttttt ttttttttt tttaatgcat gtagagggat atacactaca gtcaagatcg     1136
118 gaattggaga ttagctcata gaaatggtaa ctgcccaaga aacaaaaaaa gaaatgacta     1196
120 acaaatgggc aataatacca tcaatacctt gtcataccta tttgaatgga gaaataactc     1256
122 agttaagctc agtactggca taagcatggg gaaaatattt caattaatca gttcgagtag     1316
124 atatgttttc caaattgata gcgatattag acatttcata ttgaaattta caggtacaaa     1376
126 tataatctca gttaatgcgt agaatgcgtt tgcaattgaa caaatttaac gttttatagc     1436
128 aagaacttag gaacaaaagt tgtaatcgct tattctataa taatgttaac taaagccaaa     1496
130 gcaagtagat cggttgtata actcatttct gctatagata agtcttgact tgtatcagta     1556
132 ctgagctata aattggtcca tatatacgta gccaggcctg gccacatata gaggttaata     1616
134 aacgttatgt actgcaaaag gatagttgaa accatagcta aacgatagtc gatgtaccaa     1676
136 ccacactcca ctccaatcca atccaatcca atccactcaa atcaattcaa aacaacacac     1736
138 tcgtaaggga cacacactca cgcataatac gggacccact tcagtagaaa gtcacacgat     1796
140 atcagcgatc acggatcacg aattacggat cacggaatac gtatcacgga tcacacaggc     1856
142 ggctcacctc accaagctca gcagcaaact caccccacct aggacactgc ttccaggcag     1916
144 ctagcgaacg ctcaccaac tacaataatt agccaaccct agagtaatca gtttaccagt     1976
146 aaacagtaac cagtaactag taaccaatta ccaattacca gtaacccatc caaggagtat     2036
148 acccccctg caaacgggga agcggataaa tgtcactaga attcagcatc atcagattga     2096
150 atcacacaca atcctagtcg cctcacgcga agagaactat gtcatgatca gatatcggtg     2156
152 tatgcattct atattatgta cttcgaaata tgtaatttat taagttttcg ctatactttt     2216
154 cattcaaatt ggcaaaaacc aattcaaagg ttttcaatat tttcgaaaag cattttaggc     2276
156 tttctatgta acgtatgttt ttcaaacaaa atattagttt ttgaaacttt attatcggat     2336
158 aaacaaatgt aagccaaatt acaacgttta tgatactcca aagattcgca ctataaagtg     2396
160 gcctaaaaat agctgacgca ttagccatag gcgcttcgct tctcaagata aaacctgggc     2456
162 gtgctcaact caagaacaaa tatgtggtta tatacatata tacatatatg gggcatataa     2516
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RAW SEQUENCE LISTING DATE: 07/15/2001
PATENT APPLICATION: US/09/700,869 TIME: 20:15:54

Input Set : A:\PUR-92SEQ.doc
Output Set: N:\CRF3\07152001\I700869.raw

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168 agaggacgag agagagagag agagaaagtg aaagtgaaag ggagagatag taaaactgca     2696
170 tctgcatcca aagacacgag aattgaattc atcaataata acatacgtat aaacgatatg     2756
172 catacgatat agaattgaat ctgtaactga tgggcatata ccgcatatat atcttatata     2816
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197 gtcggcactg atggccaatc tggccgatgt ggtcaaggag gccaaggatg aggagatccc     180
199 gatgcccaaa tcgaatgact tcttcgagtc caagaccttc cgcttgctca ccctgatgct     240
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203 ggattcacgc atgccgccgc tgcccgtgtt caagcacacg catccgattg gctaggatcg     360
205 gatggctatc atttagttag ccatccatct cccagcgaca tcagtgaccg gaattggata     420
207 aaattgtgat acccgagaaa atagccattc gcctcctgaa ctcatcggaa gtgaccgcgg     480
209 agcagttcta caagcacatc ctcgagcagt accgcatcct cagccac                  527
212 atg caa cag cag cgc cag caa ctg ctg cag cgc caa cat ctc caa ctg       575
213 Met Gln Gln Gln Arg Gln Gln Leu Leu Gln Arg Gln His Leu Gln Leu
214 1               5                  10                  15
217 cag cag ctg gag gca aac aat cgc ttc cag gag gtc ttt gcc acg gcc       623
218 Gln Gln Leu Glu Ala Asn Asn Arg Phe Gln Glu Val Phe Ala Thr Ala
219             20                  25                  30
221 acc atc att cag gca cat ccg cat ccc cat cca cat ccc agg gag ccg       671
222 Thr Ile Ile Gln Ala His Pro His Pro His Pro His Pro Arg Glu Pro
223         35                  40                  45
225 ccc aag aag ccg ctt tta gga cca tat agc ccg caa ccc ggc aac ata       719
226 Pro Lys Lys Pro Leu Leu Gly Pro Tyr Ser Pro Gln Pro Gly Asn Ile
227     50                  55                  60
229 agt cac gct atg ggt ggt gat cag ttg gat gca gaa acg gaa cag ggt       767
230 Ser His Ala Met Gly Gly Asp Gln Leu Asp Ala Glu Thr Glu Gln Gly
231  65                  70                  75                  80
233 cac atg cct cta atc ctg gat acc tca ccg ccg gtc gaa gta acc gga       815
234 His Met Pro Leu Ile Leu Asp Thr Ser Pro Pro Val Glu Val Thr Gly
235                 85                  90                  95
237 atg ggt cac ctg aag cgg aag aca cat cgc ggt cac tac aaa cat cat       863
238 Met Gly His Leu Lys Arg Lys Thr His Arg Gly His Tyr Lys His His
239             100                 105                 110
241 aga gcc cga gcc ggt ggt caa aag aaa ctg tcc att gcc aat tcg atg       911
242 Arg Ala Arg Ala Gly Gly Gln Lys Lys Leu Ser Ile Ala Asn Ser Met
243         115                 120                 125
245 gcc agc tcc acg ccg agc acc aca gcc gga gga gat gcg tca atg gcc       959
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RAW SEQUENCE LISTING DATE: 07/15/2001
PATENT APPLICATION: US/09/700,869 TIME: 20:15:54

Input Set : A:\PUR-92SEQ.doc
Output Set: N:\CRF3\07152001\I700869.raw

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254 Ala Ala Gly Thr Ile Val Gln Ala Pro Gln Leu Gln Leu Tyr Thr Ser
255                 165                 170                 175
257 atg ccc att cca ctg atc ctg agt ccc agc gac gaa aag cgt cct tcg      1103
258 Met Pro Ile Pro Leu Ile Leu Ser Pro Ser Asp Glu Lys Arg Pro Ser
259             180                 185                 190
261 cac cac gcc cac gga cat gtc cat ggc gag agg cgg aac ggg gcg caa      1151
262 His His Ala His Gly His Val His Gly Glu Arg Arg Asn Gly Ala Gln
263         195                 200                 205
265 tcc ggc ggc cgg cga agg acc acg acg gca tcg gtt tct ggc tac gag      1199
266 Ser Gly Gly Arg Arg Arg Thr Thr Thr Ala Ser Val Ser Gly Tyr Glu
267     210                 215                 220
271 gcg cag acc tac ctc aat ccg ttt ctc acc ggc gag ctg atc ttc gag      1247
272 Ala Gln Thr Tyr Leu Asn Pro Phe Leu Thr Gly Glu Leu Ile Phe Glu
273 225                 230                 235                 240
275 aag taagggactg cacccagatc aggaaacgtc gcggttcatt gttttttttt           1300
276 Lys
278 ttttttttt tttaatgcat gtagagggat atacactaca gtcaagatcg gaattggaga      1360
280 ttagctcata gaaatggtaa ctgcccaaga aacaaaaaaa gaaatgacta acaaatgggc      1420
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302 acggatcacg aattacggat cacggaatac gtatcacgga tcacacaggc ggctcacctc      2080
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320 aagccaaatt acaacgttta tgatactcca aagattcgca ctataaagtg gcctaaaaat      2620
322 agctgacgca ttagccatag gcgcttcgct tctcaagata aaacctgggc gtgctcaact      2680
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330 agagagagag agagaaagtg aaagtgaaag ggagagatag taaaactgca tctgcatcca      2920
332 aagacacgag aattgaattc atcaataata acatacgtat aaacgatatg catacgatat      2980
334 agaattgaat ctgtaactga tgggcatata ccgcatatat atcttatata ccgcatatat      3040
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RAW SEQUENCE LISTING
PATENT APPLICATION: US/09/700,869

DATE: 07/15/2001
TIME: 20:15:54

Input Set : A:\PUR-92SEQ.doc
Output Set: N:\CRF3\07152001\I700869.raw

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TO/700,869

VERIFICATION SUMMARY DATE: 07/15/2001
PATENT APPLICATION: US/09/700,869 TIME: 20:15:55

Input Set : A:\PUR-92SEQ.doc
Output Set: N:\CRF3\07152001\I700869.raw

L:1 M:259 W: Allowed number of lines exceeded, (1) GENERAL INFORMATION:
L:13 M:270 C: Current Application Number differs, Replaced Current Application Number
L:15 M:271 C: Current Filing Date differs, Replaced Current Filing Date

RECEIVED
JUL 25 2001
TECH CENTER 1600/2900

SEQUENCE LISTING

<110> Pak, William L.
Li, Chenjian
Geng, Chaoxian

<120> Calcium Channel Regulators

<130> 7024-384/PUR-92

<140> Unknown

<141> 1999-05-18

<150> U.S. 60/087,368; U.S. 60/098,072

<151> 1998-05-18; 1998-08-27

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catcctcagc cac atg caa cag cag cgc cag caa ctg ctg cag cgc caa cat 352
Met Gln Gln Gln Arg Gln Gln Leu Leu Gln Arg Gln His
1 5 10

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Leu Gln Leu Gln Gln Leu Glu Ala Asn Asn Arg Phe Gln Glu Val Phe
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gcc acg gcc acc atc att cag gca cat ccg cat ccc cat cca cat ccc        448
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agg gag ccg ccc aag aag ccg ctt tta gga cca tat agc ccg caa ccc        496
Arg Glu Pro Pro Lys Lys Pro Leu Leu Gly Pro Tyr Ser Pro Gln Pro
                50                  55                  60

ggc aac ata agt cac gct atg ggt ggt gat cag ttg gat gca gaa acg        544
Gly Asn Ile Ser His Ala Met Gly Gly Asp Gln Leu Asp Ala Glu Thr
            65                  70                  75

gaa cag ggt cac atg cct cta atc ctg gat acc tca ccg ccg gtc gaa        592
Glu Gln Gly His Met Pro Leu Ile Leu Asp Thr Ser Pro Pro Val Glu
        80                  85                  90

gta acc gga atg ggt cac ctg aag cgg aag aca cat cgc ggt cac tac        640
Val Thr Gly Met Gly His Leu Lys Arg Lys Thr His Arg Gly His Tyr
    95                  100                 105

aaa cat cat aga gcc cga gcc ggt ggt caa aag aaa ctg tcc att gcc        688
Lys His His Arg Ala Arg Ala Gly Gly Gln Lys Lys Leu Ser Ile Ala
110                 115                 120                 125

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Asn Ser Met Ala Ser Ser Thr Pro Ser Thr Thr Ala Gly Gly Asp Ala
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Ser Met Ala Thr Ala Ala Thr Leu Pro His Gly Tyr Met Asp Ala Pro
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cta aat ccg gcg gca gga acc att gtc cag gca cca caa ctg cag cta        832
Leu Asn Pro Ala Ala Gly Thr Ile Val Gln Ala Pro Gln Leu Gln Leu
        160                 165                 170

tac acc tcg atg ccc att cca ctg atc ctg agt ccc agc gac gaa aag        880
Tyr Thr Ser Met Pro Ile Pro Leu Ile Leu Ser Pro Ser Asp Glu Lys
    175                 180                 185

cgt cct tcg cac cac gcc cac gga cat gtc cat ggc gag agg cgg aac        928
Arg Pro Ser His His Ala His Gly His Val His Gly Glu Arg Arg Asn
190                 195                 200                 205

ggg gcg caa tcc ggc ggc cgg cga agg acc acg acg gca tcg gtt tct        976
Gly Ala Gln Ser Gly Gly Arg Arg Arg Thr Thr Thr Ala Ser Val Ser
                210                 215                 220

ggc tac gag gcg cag acc tac ctc aat ccg ttt ctc acc ggc gag ctg       1024
Gly Tyr Glu Ala Gln Thr Tyr Leu Asn Pro Phe Leu Thr Gly Glu Leu
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Ile Phe Glu Lys
240

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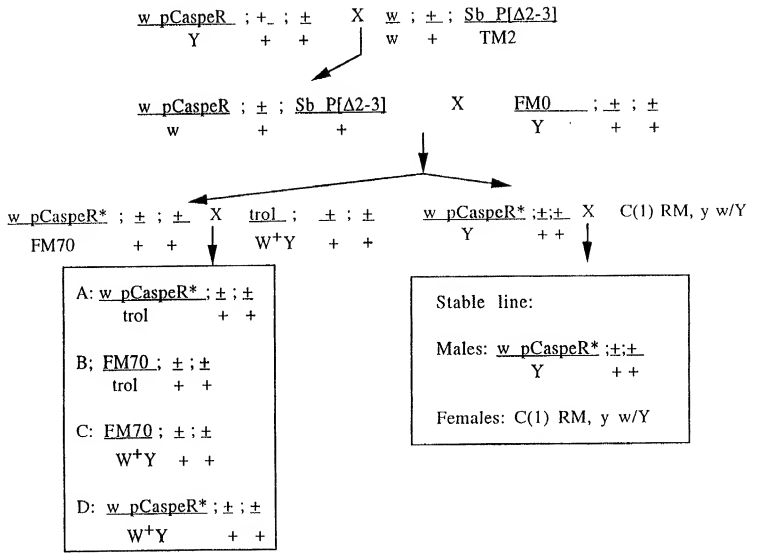
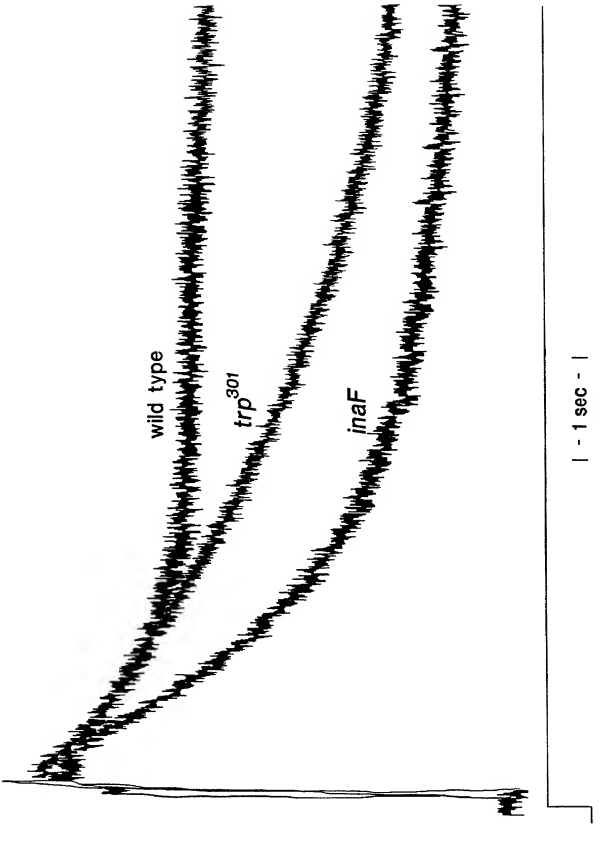


Fig. 1

WO 99/60022
PCT/US99/10827

09700869

Fig. 2



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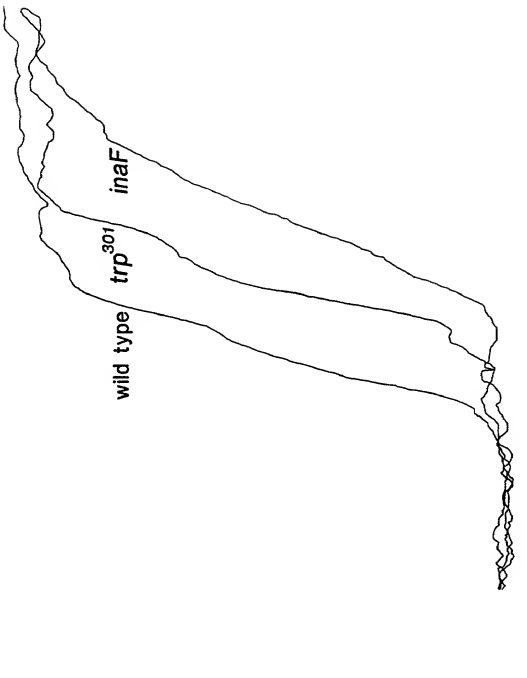
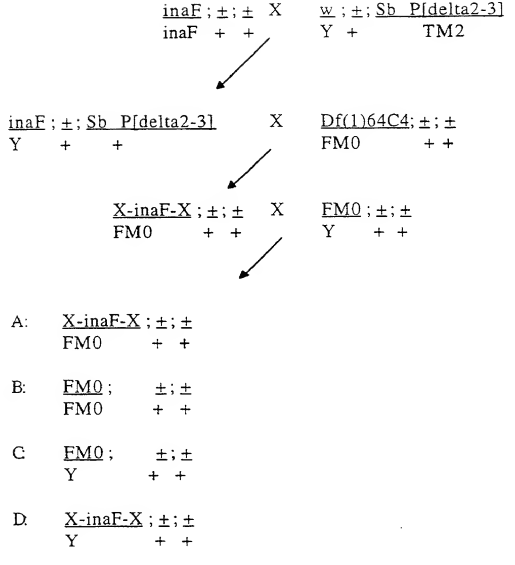


Fig. 4

Fig. 5A

inaF;bw;st 19 days old
Light/Dark reared

Fig. 5B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X-inaF-X ; ± ; ± X FM0 ; ± ; ±
FM0 + + Y + +
A: X-inaF-X ; ± ; ±
FM0 + +
B: FM0 ; ± ; ±
FM0 + +
C FM0 ; ± ; ±
Y + +
D X-inaF-X ; ± ; ±
Y + +

Fig. 6

WO 99/09032
8/14
09/508869
PCT/US98/16822

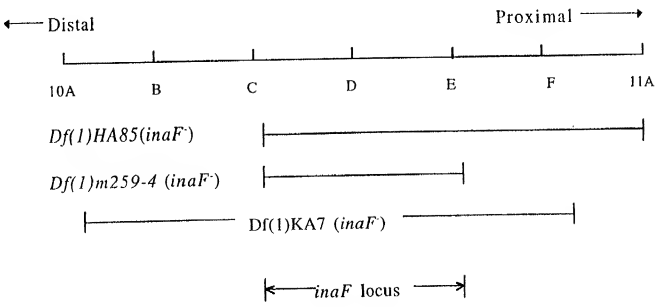

Fig. 7

Genomic Southern for RFLP Analysis
EcoRI BamHI HindIII
1 2 3 4 5 6 7 8 9 10 11 12
• 1,5,9—wild type
• 2,6,10-mutator
• 3,7,11-Jumpstarter
• 4,8,12-inaF

Fig. 8

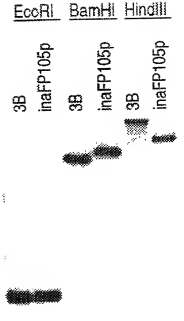
EcoRI
BamHI
HindIII
3B
inaFP105p
3B
inaFP105p
3B
inaFP105p

Fig. 9

Fig. 10

Northern Analysis
Genomic clone A23 detects an eye specific transcript which is absent in InaF mutant
wild type head
wild type body
InaF head
eye head
RP49 Control

Fig. 11

6.7 kb
3.5 kb
0.6 kb
R
R
R
Composite
A23 clone
P element
inaF^P105p
inaF^P106x
inaF^P111x
5'
3'
inaFcDNA
1 kb

Fig. 12

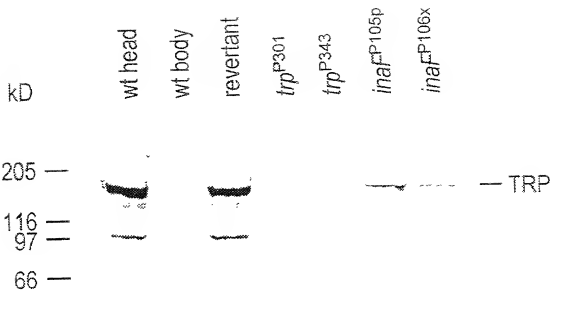
kD
wt head
wt body
revertant
trpP301
trpP343
inaFP105p
inaFP106x
205
116
97
66
TRP

Fig. 13

#6

JUL 03 2001

Type a Plus sign (+) inside this box →

JJS:132117:jj

WENMM SB/01 (4-99)

DECLARATION FOR UTILITY OR DESIGN PATENT APPLICATION (37 CFR 1.63)

Attorney Docket Number	7024-491
First Named Inventor	William L. Pak et al.
COMPLETE IF KNOWN	
Application Number	09/700,869
Filing Date	November 20, 2000
Group Art Unit	Unknown
Examiner Name	Unknown

☐ Declaration Submitted With Initial Filing **OR** ☒ Declaration Submitted after Initial Filing (surcharge 37 CFR 1.16 (e) required)

As a below named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first, and sole inventor (if only one name is listed below) or an original, first, and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

CALCIUM CHANNEL REGULATORS

(Title of the Invention)

the specification of which

☐ is attached hereto **OR** ☒ was filed on (MM/DD/YYYY) 11/20/2000 as United States Application Number 09/700,869 and was amended on (MM/DD/YYYY) _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to above.

I acknowledge and hereby disclose information which is material to patentability as defined in 37 CFR 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. 119 (a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one country other than the United States of America, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate, or of any PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application Number(s)	Country	Foreign Filing Date (MM/DD/YYYY)	Check Only if Priority Not Claimed	Certified Copy Attached? YES	NO
			☐	☐	☐

☐ Additional foreign application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto:

I hereby claim the benefit under 35 U.S.C. 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)
60/087,368	05/18/1998
60/098,072	08/27/1998

☐ Additional provisional application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

JUL 03 2001 PATENT & TRADEMARK OFFICE

Type a plus sign (+) inside this box → [+]

DECLARATION – Utility or Design Patent Application

I hereby claim the benefit under 35 U.S.C. 120 of any United States application(s), or 365(c) of any PCT international application designating the United States of America, listed below, and insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 CFR 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

U.S. Parent Application or PCT Number	Parent Filing Date (MM/DD/YYYY)	Parent Patent Number (if applicable)
PCT/US99/10821	05/18/1999	

[ ] Additional U.S. or PCT international application numbers are listed on a supplemental priority data sheet PTO/SB/02B attached hereto.

As a named inventor, I hereby appoint the following registered practioner(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

[ ] Customer Number [ ] → *Place Customer Number Bar Code Label Here*

OR

[X] Registered practioner(s) name/registration number listed below.

Name	Registration Number	Name	Registration Number
Jason J. Schwartz	43,910		

[X] Additional registered practioner(s) named on supplemental Registered Practioner Information sheet PTO/SB/02C attached hereto.

Direct all correspondence to: [ ] Customer Number Bar Code Label [ ] *OR* [X] Correspondence address below

Name	Jason J. Schwartz						
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Address	Bank One Center/Tower, Suite 3700, 111 Monument Circle						
City	Indianapolis			State	IN	ZIP	46204-5137
Country	US	Telephone	317/ 634-3456			Fax	317/637-7525

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Name of Sole or First Inventor: [ ] A petition has been filed for this unsigned inventor.

Given Name (first and middle [if any])	Family Name or Surname
William L.	Pak

Inventor's Signature								Date	6/25/01
Residence: City	West Lafayette	State	IN	Country	USA			Citizenship	USA
Post Office Address	1025 Windowood Lane								
Post Office Address									
City	West Lafayette	State	IN	ZIP	47906	Country	US		

[X] **Additional inventors are being named on the 2 supplemental Additional Inventor(s) sheet(s) PTO/SB/02A attached hereto.**

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JUL 03 2001
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Name of Joint Inventor, if any:			A petition has been filed for this unsigned inventor.					
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Chenjian			Li					
Inventor's Signature	Chenjian Li						Date	6/28/01
Residence: City	Tarrytown New York	State	NY	Country	USA		Citizenship	CN
Post Office Address	221 Carrollwood Drive	503 East 63rd St, Apt 14R						
Post Office Address								
City	Tarrytown New York	State	NY	ZIP	10591 10021	Country	USA	

Name of Joint Inventor, if any:			A petition has been filed for this unsigned inventor.					
Given Name (first and middle [if any])			Family Name or Surname					
Chaoxian			Geng					
Inventor's Signature							Date	
Residence: City	West Lafayette	State	IN	Country	USA		Citizenship	CN
Post Office Address	1904 Willet Court							
Post Office Address								
City	West Lafayette	State	IN	ZIP	47906	Country	USA	

Name of Joint Inventor, if any:			A petition has been filed for this unsigned inventor.					
Given Name (first and middle [if any])			Family Name or Surname					
Inventor's Signature							Date	
Residence: City		State		Country			Citizenship	
Post Office Address								
Post Office Address								
City		State		ZIP		Country		

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JUL 03 2001
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ADDITIONAL INENTOR(S)
Supplemental Sheet
Page ___ of _____

Name of Joint Inventor, if any:								A petition has been filed for this unsigned inventor.	
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Chenjian						Li			
Inventor's Signature								Date	
Residence: City	Tarrytown		State	NY	Country	USA		Citizenship	CN
Post Office Address	221 Carrollwood Drive								
Post Office Address									
City	Tarrytown	State	NY	ZIP	10591	Country	USA		

Name of Joint Inventor, if any:								A petition has been filed for this unsigned inventor.	
Given Name (first and middle [if any])						Family Name or Surname			
Chaoxian						Geng			
Inventor's Signature								Date	06/29/01
Residence: City	West Lafayette		State	IN	Country	USA		Citizenship	CN
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City	West Lafayette	State	IN	ZIP	47906	Country	USA		

Name of Joint Inventor, if any:								A petition has been filed for this unsigned inventor.	
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City		State		ZIP		Country			

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